Translocation of Neonicotinoid Insecticides From Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees

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ABSTRACT The death of honey bees, *Apis mellifera* L., and the consequent colony collapse disorder causes major losses in agriculture and plant pollination worldwide. The phenomenon showed increasing rates in the past years, although its causes are still awaiting a clear answer. Although neonicotinoid systemic insecticides used for seed coating of agricultural crops were suspected as possible reason, studies so far have not shown the existence of unquestionable sources capable of delivering directly intoxicating doses in the fields. Guttation is a natural plant phenomenon causing the excretion of xylem fluid at leaf margins. Here, we show that leaf guttation drops of all the corn plants germinated from neonicotinoid-coated seeds contained amounts of insecticide constantly higher than 10 mg/l, with maxima up to 100 mg/l for thiamethoxam and clothianidin, and up to 200 mg/l for imidacloprid. The concentration of neonicotinoids in guttation drops can be near those of active ingredients commonly applied in field sprays for pest control, or even higher. When bees consume guttation drops, collected from plants grown from neonicotinoid-coated seeds, they encounter death within few minutes.

KEY WORDS guttation, neonicotinoid, honey bee, seed coating

Phytophagous insects occurring in soil at sowing time tend to concentrate around on corn, *Zea mays* L., seedlings causing extensive damage. Granular insecticides applied to the soil have been the method of choice for their control for a long time. More recently, the strategy has been surpassed by the seed coating technique using neonicotinoids, which are active against a broad range of pest species, including wireworms (*Agriotes* spp.) and the rootworm *Diabrotica virgifera virgifera* LeConte (Altmann 2003).

Among the main reasons of success of neonicotinoids is their systemic action. Upon application on the seed surface, the active compound is translocated and distributed throughout the whole plant, conferring a substantial and long-lasting control of insects and protecting young plants also from sucking leafhoppers and aphids (Magalhaes et al. 2009), which are potential vectors of plant virus (Maienfisch et al. 2001). Nowadays, neonicotinoids are widely used for seed treatment in cotton (*Gossypium* spp.), sugarbeet (*Beta vulgaris* L.), oilseed rape (*Brassica napus* L.) corn (*Zea mays* L.), and other cereals and crops (Elbert et al. 2008). The reduced load of insecticide per field unit, allowed by confining it on the seed, represents main advantages in environmental terms compared with former products requiring whole-soil or furrow applications. Elbert et al. (2008) pointed out impressive figures revealing the turnover toward insecticidal seed treatment. Starting from: a niche-level market of €155 million in 1990, mostly represented by carbamates, by 2005 seed coating developed into a €535 million market, with a 77% share for neonicotinoid insecticides.

The loss of pollinating bees is a worldwide crisis. In particular it became manifest as colony collapse disorder (CCD), characterized by a sudden disappearance of worker bees that do not return to the hive. Parasitic mites and viruses have come under suspicion, although no clear conclusions could be drawn as concerns these biotic causes. Pesticides have been shown to be more directly involved and in recent years the attention has been focused on neonicotinoids (imidacloprid, clothianidin, and thiamethoxam), a class of insecticides among the most widely used worldwide. The effects of neonicotinoids, such as imidacloprid on honey bees (Suchail et al. 2000, Maus et al. 2003) could be consistent with the symptoms of CCD. However, the blame on neonicotinoids has not yet been conclusive as the amounts detected in nectar and pollen of plants grown from treated seeds were lower than 10 ng/g (10 ppb), whereas higher doses, as >40 μg/liter (40 ppb) are necessary for abnormal honey bee foraging behavior, >0.5 mg/liter (0.5 ppm) for the first missing bees, and >3 mg/liter (3 ppm) for 100% of the

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bees failing to return to a source of sugar offered to them (Yang et al. 2008).

In Italy, a highly recurring coincidence between corn sowing time and bee death has been noticed previously (Greatti et al. 2003, Greatti et al. 2006), leading to the hypothesis that solid coating debris and dust uplifted from sowing machines could fall over nearby vegetation and contaminate wildflowers.

Within the same frame of thought, we postulated that a different and hitherto overlooked source of directly lethal doses could be present in the fields, and we took into consideration the hypothesis that intoxicating concentrations could accumulate in guttation drops of young corn plants.

Guttation (from the Latin "gutta"=drop) is the formation of drops of xylem sap on the tips or along the edges of leaves. It is a physiological phenomenon occurring in many vascular plants, in particular grasses, water entering roots creates a slight pressure that forces it to rise and be exuded through the hydatodes at leaf margins. This is a regular occurrence in many plants and is not restricted to nighttime, although in the dark stomatal closure can lead to higher internal pressure that increases guttation drop volumes, thereby enhancing the visibility of the phenomenon (Goatley and Lewis 1966, Koulman et al. 2007). Guttation is common but often unnoticed as easily confused with dew characterized by small condensation drops from atmospheric humidity. Guttation drop can roll off, evaporate or may be sucked back into the leaf (Chen and Chen 2007).

Bees require intense drinking activity (Visscher et al. 1996, Kühnholz and Seeley 1997) and have been reported to collect guttation water (Shawki et al. 2005).

In the current study, we wanted to verify whether neonicotinoids used for seed coating could be translocated in guttation drops and reach concentrations toxic to bees. In parallel, we tested the toxicity of serial concentrations of these insecticides by setting up a test apt to evaluate, in reasonably short time, the appearance of intoxication symptoms in bees upon consumption of neonicotinoid aqueous solutions.

Materials and Methods

Insect Origin. Trials were carried out in the experimental farm of the faculty of Agriculture (University of Padova) located in Legnaro, Italy. Bees (*Apis mellifera* L.) used for the tests were collected from different colonies residing within the farm's field facilities. When season allowed the bees to fly, they were collected with a net in front of the hive; otherwise (in winter), bees were collected from within the hive.

Insecticides Tested. Trials started in spring 2008. Guttation drops were collected from corn seedlings germinated from commercial seeds coated with the neonicotinoids imidacloprid (Gaucho 350 FS, Bayer Cropscience; 0.5 mg/seed), clothianidin (Poncho, Bayer Cropscience AG, Leverkusen, Germany; 1.25 mg/seed), thiamethoxam (Cruiser 350 FS, Syngenta International AG, Basel, Switzerland; 1 mg/seed), and fipronil (Regent 500 FS, BASF SE, Ludwigshafen, Germany; 1 mg/seed). The last nonsystemic compound is a member of the phenyl pyrazole class of pesticides. Each of the four insecticides mentioned was a regularly registered product for corn seed coating in Italy in 2008. Seeds used (hybrid PR34N84) were from Pioneer Hi-Bred Italy (Johnston, IA), and all also were coated with the fungicide Celest XL (Syngenta), based on Fludioxonil (2.4%) and Metalaxyl-M (0.93%). The untreated control was also from Pioneer Hi-Bred (for biological agriculture) and belonged to the hybrid PR33A46.

In field crops, we had cases treated with each of the above-mentioned compounds. For potted plants, we focused essentially on imidacloprid.

Collection of Guttation Drops. During spring (April) corn seedlings were grown in open field, spaced 20 cm within the row and 75 cm between rows. In subsequent periods (May), tests were replicated by sowing coated seeds in pots with a diameter of 15 cm and growing two to five plants per pot in the laboratory. In total, six to eight pots for each compound were used and equal numbers were sown with uncoated seeds as control, or with seeds coated with fungicides.

For each seedling, we gathered all guttation drops at all plant levels, by using Pasteur micropipettes. Collection in the field was carried out from 8:00 to 9:00 a.m. from all plants within a row, until a volume of 5 ml was available. In the laboratory, because guttation occurs throughout the days and night, it was possible to collect them three times a day, yielding a volume of ≈1–2 ml/d. Samples were stored at +2°C. Half of the volume was sent for chemical analyses and half for toxicity bioassays, which were normally performed within 2–3 d.

Collection of guttation drops was carried out from corn emergence up to the first 3 wk for each of the treatments as subsequently the phenomenon ceased in its intensity both in the field and in the laboratory.

Insecticide Content in Guttation Drops. Pure chemicals for the preparation of solutions of thiamethoxam, clothianidin, imidacloprid, and fipronil, to be used for reference toxicity curves and as analytical determination standards, were from Fluka (Sigma-Aldrich Group, Milan, Italy; Pestanal, purity >99.7% for thiamethoxam, clothianidin, imidacloprid, >97.6% for fipronil). Methanol (VWR, International, Milan, Italy), and acetonitrile (Riedel de Haën, Sigma-Aldrich Group) were of high-performance liquid chromatography (HPLC) grade. Pure water was produced by Milli-Q equipment (Millipore, Billerica, MA). HPLC analytical determinations were performed on a 680 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with UV-Vis diode array detector, a 20-μl sampling loop of the injector valve and an Alltech Alltima C18 analytical column (5 μm, 4.6 × 250 mm; Altech Associates, Deerfield, IL), according to published methods (Ying and Kookana 2004, Singh et al. 2004, Rancan et al. 2006, Zhou et al. 2006) optimized for different matrices. The following instrumental procedure was optimized: eluent flow rate of 1.2 ml/min, gradient elution (0–4 min, 70:30%

Fig. 1. Guttation drops on corn leaves in the field. (Online figure in color.)

water/acetonitrile; 4–9 min, linear gradient to 100% acetonitrile; 9–13 min, 100% acetonitrile), 20°C of column temperature, multiwavelength acquisition of detector signal and analytes quantification at $\lambda = 252$ nm for thiamethoxam, $\lambda = 269$ nm for clothianidin and imidacloprid, and $\lambda = 215$ nm for fipronil. Instrumental calibration (external) was performed by analysis of standard solutions in the 0.1–100 mg/liter concentration range of analytes in methanol. The analysis of guttation solutions was performed by direct injection, after filtration on a Millex HV 0.45-μm syringe filter (diam. = 4 mm; Millipore) of 100–300 μl of the sample.

Toxicity of Guttation Drops to Honey Bees. The test was carried out at 20–22°C in a temperature-controlled room. Before the tests bees were kept in cages (20 by 20 cm) for at most 2 h without water and food.

Single bees were sampled from the cage with test tubes and introduced into 5-cm-sided cubic net (tulle) cages. After 15 min of adaptation, the guttation water was offered. We tested either plain guttation drops or guttation drops with the addition of 15% honey (vol:vol) (21%, vol:wt, according to specific weight of honey of 1.4). For bees to be attracted to drink, 30 μl was placed on the top of the net cage inside a capillary glass tube (100 mm in length with a diameter of 1 mm). Actual liquid consumption was ascertained by variation of the level in the capillary, and a drinking event was defined by the consumption of a minimum of 5 μl of liquid (bees that did not accept to drink within 5 min were discarded).

After 20 min from solution consumption, a drop of honey was offered on the top of the cage to feed bees. The bee was constantly observed and from the first event of drinking, that normally occurred shortly after offering the capillary with solution, we recorded the time required for the appearance of two intoxication symptoms that always occurred before death. The first was a jerky inward arching of the abdomen, and the second was a definitive block of the flight capability caused by a paralysis of the thorax muscle and therefore of the wings.

Evaluation of Dose–Response Effect. To observe the relationship between concentration and response of the above-mentioned two intoxication symptoms, we offered bees with solutions of pure insecticides in water with 15% honey, at increasing insecticide concentrations using the same method described above for guttation drops. We started with concentrations of 100 mg/liter with progressive halving, up to dilutions no longer causing, within 1 h, the two intoxication

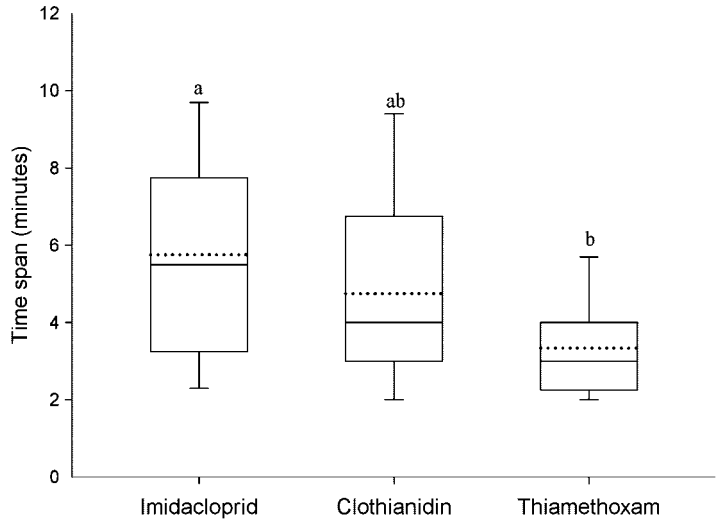

Fig. 2. Time between appearance irreversible wing-block and drinking of guttation drops collected on leaves of field corn crops, from three marketed neonicotinoid-coated. Guttation sampled on plants germinated from untreated seeds did not show any toxicity. The whisker represents the maximum and the minimum of the recorded time; the dotted line indicates the average; the upper, middle, and lower lines of the box indicate the 75, 50, and 25% of the time, respectively. Bars marked with different letters indicate significant differences ($P < 0.05$; Tukey–Kramer test).

symptoms in all bees which had drink at least 5 μl. We tested each dosage, for each of the three neonicotinoids (imidacloprid, clothianidin, and thiamethoxam). We also assayed a saturating dose (3.8 mg/liter) of the non-neonicotinoid fipronil. Each treatment was repeated on a minimum of 12 bees, separately tested, for each concentration. The actual concentration of insecticide in the solutions, obtained by theoretical dilution, was confirmed by chemical analysis.

Statistical Analysis. The time between drinking from guttation drops (from three marketed neonicotinoid-coated seeds) and the appearance of intoxication symptom, as well as different concentrations of chemicals in guttation samples were compared by one-way variance analysis (ANOVA). Subsequently, a significance difference test (Tukey–Kramer) was applied.

Results and Discussion

Collection of Guttation Drops. First, we observed that the totality of corn plants in the field feature abundant and continuous guttation drops during their first 3 wk of growth (Fig. 1). Although the guttation water tends to evaporate during the warmer hours of the day, in corn seedlings, guttation drops can flow down into the crown cup and persist drinkable for most of the day. Although textbook definitions tend to relate guttation to conditions of moist soil and humid air, the phenomenon is not restricted by these parameters that only enhance the size of the drops facilitating their observation. Moreover, guttation drops formed under conditions of lower soil moisture and dryer air can contain even more concentrated solutes as a consequence of the progressive water evaporation. During April–May, guttation drops were regularly found on vegetation until 9–10 a.m. Only on particularly windy days drops were not found. From potted plants in the lab the collected amount was 30–150 μl/d per plant, whereas in the field a single collection in the morning easily allowed to gather 1–3 ml from 100 plants.

Insecticide Content in Guttation. Chemical analyses of the guttation water from laboratory-grown corn plants during 3 wk of growth showed the presence of the corresponding seed coating neonicotinoids in all samples. Guttation drops collected on plants from neonicotinoid-coated seeds contained concentrations of each respective active ingredient of (mean $\pm$ SE) 47 ± 9.96 mg/liter for imidacloprid, 23.3 ± 4.2 mg/liter for clothianidin, and 11.9 ± 3.32 mg/liter for thiamethoxam with statistically significant differences (ANOVA: $F = 7.51$; df = 2, 15; $P = 0.005$). The amount of imidacloprid found in drops of plants grown from seeds treated with 0.5 mg per seed was significantly more concentrated than that of thiamethoxam in guttation drops of plants treated with 1 mg of active ingredient per seed ($P < 0.01$; Tukey–Kramer test). The nonsystemic fipronil was never found above its detection limit in guttation water.

The higher translocation from seed to guttation observed for imidacloprid is surprising in light of its lower amount in the coating compared with thiamethoxam and clothianidin. In another experimental analysis carried out on drops produced from individually potted plants obtained from seeds coated with imidacloprid average concentrations resulted of 82.8 ± 14.07 mg/liter, with maxima reaching over 110 mg/liter. Therefore, as first approach neonicotinoids concentration on guttation does not seem strictly dependent on density of plants per pot.

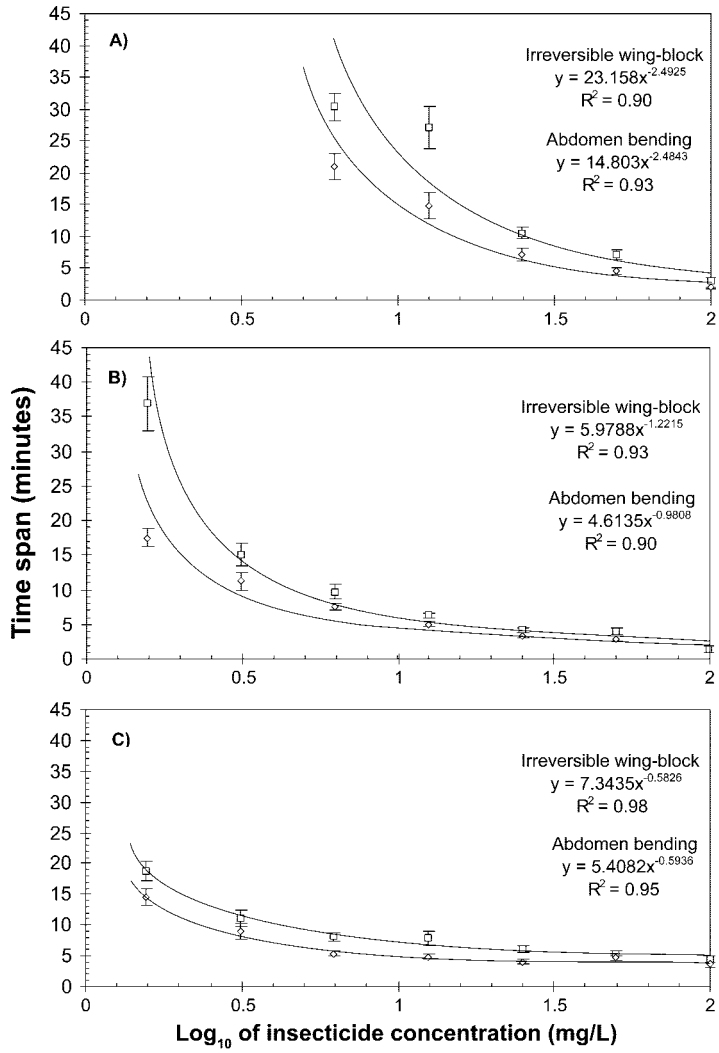

Fig. 3. Toxicity of neonicotinoid imidacloprid (A), clothianidin (B), and thiamethoxam (C) on honey bees. Neonicotinoid toxicity was calibrated as time of appearance after drinking of two poisoning symptoms (squares, irreversible wing-block; diamonds, abdomen bending) upon offering bees drops of water with 15% honey containing insecticides (pure chemical). Each symbol represents the mean of 12 replicates, and the vertical bars indicate the standard error of the means. Concentration data (milligrams per liter) are transformed in $\log_{10}$.

Neonicotinoid concentration in guttation drops resulted in general rather variable presumably due to environmental factors as concentration via water evaporation, collection time of the day, and time elapsed since seedling emergence.

In more recent experimentation for all three neonicotinoids peak concentrations above 100 mg/liter were observed and also >200 mg/liter for imidacloprid. These values are near or even higher than those of insecticides commonly applied in field sprays for pest control. Regardless, insecticide translocation from seed and accumulation in guttation seems rather clear and efficient.

Toxicity of Guttation Drops to Honey Bees. After consumption of the toxic drops, the first noticeable effect was a generic agitation similar to that occurring upon starvation. The first objective intoxication symptom was an arching of the abdomen (probably a stinging reflex). At this stage, the insect still retains its flying capability and profuse regurgitation events can often be seen. Subsequently, the bee undergoes wing paralysis and uncoordinated movements. The latter event constantly resulted an irreversible stage for all the tested guttation, thus constituting an objectively recordable cue of the subsequent lethality. Death, as defined by complete stillness, was not plotted because the time between wing block and possible residual capability to move a leg resulted extremely variable, as reported by Suchail et al. (2000). The test also enabled us to ascertain whether single bees does actually take up the solution offered and at which volume, with good approximation.

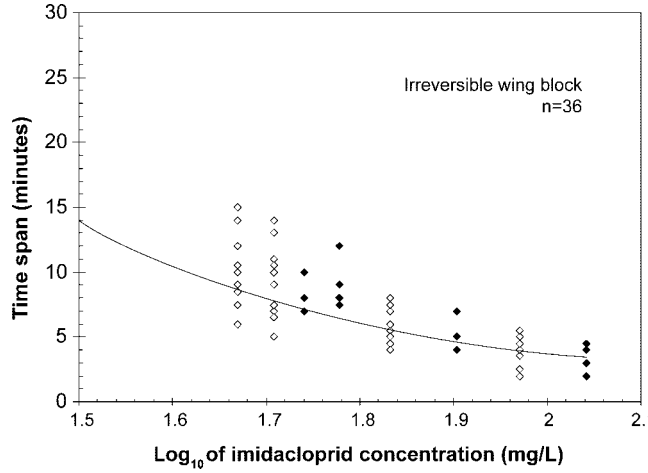

Fig. 4. Time interval between appearance irreversible wing-block of single caged bees and ingestion of guttation drops collected from leaf of potted (1–20-d-old) corn seedlings from imidacloprid-coated seeds. Concentration was determined by HPLC analysis. The curve corresponds to that shown in Fig. 3A for pure imidacloprid at the higher doses. Black symbols, pure guttation; white symbols, guttation with 15% honey. Concentration data (milligrams per liter) are transformed in $\log_{10}$.

Guttation drops collected in the field on plants grown from commercial seeds coated with the three neonicotinoids considered, offered to bees without honey, caused wing block in a time ranging between 2 and 9 min from consumption (Fig. 2). Those from plants whose seeds were coated with thiamethoxam resulted significantly more toxic in comparison with the imidacloprid-coated seeds (ANOVA: $F = 3.71$; df = 2, 33; $P = 0.035$). Control guttation drops from noncoated seeds or coated with fungicides did not display toxicity. Guttation drops from plants whose seeds were coated with the nonsystemic insecticide fipronil resulted less toxic or not consistently lethal (data not shown). Thirsty bees consumed immediately the field-collected drops offered in the cage top but often bees need long periods before drinking causing delays in the test. For such reason, for guttation drops collected from potted plants we added 15% honey to the drops to promptly attract bees to drink. This honey concentration was the minimum ensuring solution uptake within 5 min, a time compatible with the efficiency of the test.

In agreement with data from field-collected guttation drops, toxicity of guttation from potted plants germinated in the laboratory from neonicotinoid-coated seeds, irrespective to 15% honey addition, showed a strict correlation between active compound concentration and toxicity. In particular for imidacloprid the totality of bees ($n = 63$) that ingested guttation fluid underwent irreversible wing paralysis within a few minutes. Both with pure guttation drops and with those with 15% honey, the wing block symptom was in a range of 2–4 min for concentrations above 100 mg/liters and of 6–15 min for those around 50 mg/liters. Guttation toxicity seems clearly related to the neonicotinoid content.

Preliminary tests carried out by offering bees guttation drops of plants from clothianidin or thiamethoxam-coated seeds showed that wing block occurs within shorter times compared with imidacloprid at corresponding concentrations (data not shown). This would confirm that clothianidin and thiamethoxam are more toxic than imidacloprid, although less concentrated in guttation drops, as indicated above. Also, in potted plants, guttation drops from control, untreated seeds plants were harmless to bees.

Evaluation of Dose–Response Effect. The test devised to verify whether insecticides in water solution with 15% honey could kill drinking bees in short time lapses was satisfactory and of simple setup. Few minutes after drinking from neonicotinoid solutions in lethal concentrations, an excited behavior was observed followed by abdomen bending and wing paralysis as observed for guttation. The two symptoms resulted irreversible for all the neonicotinoid under study at all dosages reported (Fig. 3).

Bees showed a different response to the three neonicotinoids. For clothianidin and thiamethoxam, at the lowest concentrations of 1.5 mg/liter ($\log_{10} = 0.18$), the chosen symptoms (abdomen bending and wing paralysis) manifested before 1 h. For imidacloprid, the same could be observed at concentrations ≥6.25 mg/liter ($\log_{10} = 0.8$), indicating a lower toxicity toward bees (Fig. 3). Increasing the dosage, the interval between abdomen bending and wing block decreased progressively, becoming nearly null at 100 mg/liter ($\log_{10} = 2$) for all neonicotinoids tested (Fig. 3). When using doses lower than the doses reported (Fig. 3), either the symptoms did not occur or they did sometimes in reversible manner and in a time exceeding 1 h. Those bees, when fed, would normally survive for at least 24 h. It must be noticed that, as it makes use of a single event of uptake, the test is less severe than those in use to evaluate the median lethal concentration (LC_{50}), for which poisoning solutions are kept available for longer time. Results are in agreement with Yang et al. (2008) who reported that the imidacloprid concentration ≥3 mg/liter in a sugar solution

is the threshold preventing bees to return to foraging. This value is close to the one (6 mg/liter) at which we observe a wing paralysis on all insects tested in <1 h.

Within each given neonicotinoid concentration, no clear relationship between the actual intake volume and time of appearance of the symptoms was noticed, presumably due to individual response variability and to the frequent regurgitation events that can bias the dose–response dependency.

No evident neonicotinoid repellency could be noticed as their concentration neither clearly deter bees from drinking, nor directly affected the volume ingested. These aspects would be the object of future studies.

The effects of pure insecticide solutions (Fig. 3) and those of guttation drops in which a corresponding concentrations was ascertained by chemical analyses, resulted in good agreement. In particular, for imidacloprid the time of appearance of the flight stop distributes with good correspondence along the curve independently obtained by tests in which pure imidacloprid serial dilutions at known concentrations were offered to bees (Fig. 4).

Therefore, the neonicotinoid content in guttation drops seems to satisfactorily explain their toxicity. No additional synergic effect of other compounds present in guttation drops seems to apply in the observed phenomena.

The presence of guttation drops on corn leaves in agricultural crops is easily observable from emergence until up to ≈3 wk. In northern Italy, this is normally coincident with times from the second week of April to mid-May. Water fetching activity can be rather intensive also in spring, bees are often seen accessing water from different sources and when ground puddles are not available, plant guttation drops represent an exploited alternative. Although, as the season unfolds, blossoming flowers can provide water-containing nectar fluids, in early periods bees cannot yet rely on these. It is to be remarked in this respect that in the past 10 yr (in which an outbreak of bee mortality has been recorded) new cold-resistant corn hybrids have been massively introduced in agriculture that allow an anticipated mid-March sowing in a time that precedes the opening of the majority of wildflowers.

Being the likelihood that bees could drink from cornfield or other crops guttation drops not yet quantified, it is still not possible to draw a judgment on a possible correlation between neonicotinoid translocation into guttation drops and CCD. Regardless, the presence of a source of water carrying in solution neonicotinoid concentrations up to the levels shown in the current study, and persisting for weeks on more than a million hectares in the sole northern Italy, is a threatening scenario that does not comply with an ecologically acceptable situation.

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